THE LINEAR REPRESENTATION OF DOSAGE-RESPONSE CURVES IN MICROBIAL-ANTIBIOTIC ASSAYS[1]

HENRY P. TREFFERS

Department of Microbiology, Yale University, New Haven, Connecticut

Received for publication December 20, 1955

The so-called turbidimetric[2] or photometric assay, in which the effect of antibiotics or other drugs on the growth of microorganisms in liquid media is measured in a photoelectric colorimeter, has many advantages, including rapidity, objectivity of numerical evaluation, definable (initial) drug concentration and an absence of diffusion effects (Heatley, 1949). The various modifications have received numerous applications, especially to the estimation of drug potencies (Joslyn and Galbraith, 1950) and to the determination of the sensitivity levels of microorganisms (Jackson and Finland, 1951). Most reports have dealt with bacteria but the methods may be extended to other organisms (fungi, protozoa, etc.) which grow well in liquid media.

The present paper is concerned with a graphical treatment of photometric assay data so that a linear representation may be made. This not only provides more economical use of all of the experimental data with ready assignment of useful endpoints, but also permits certain conclusions to be drawn concerning the comparative value of the data as well as possible changes in the mechanisms of action. The graphical method has been in routine use in our laboratory for several years, in titrations involving representative gram positive and gram negative bacteria and most of the common antibiotics. It has also led to minor modifications of the photometric assay so that fuller advantage might be taken of this treatment of the resulting data.

The basic statistical principle has been widely employed in pharmacology and entomology and linear plots for a normal deviate (or probit) response against a logarithmically graded stimulus have been found for such diverse phenomena as the killing of insects by DDT, of drosophila ova by x-ray, or the response of cats to digitalis (Finney, 1952). It has not been generally employed in microbiology, however, except for a few serological measurements (Waksman, 1949; Maaløe, 1946) and in a series of recent communications from our laboratory (Treffers *et al.*, 1953–54; Treffers and Muschel, 1954; Muschel and Treffers, 1956*a*, *b*, *c*) which deal with the antibacterial effects of antibiotics, antibody and complement, separately or in combination. The apparent unfamiliarity of many of our colleagues with the special properties and advantages of this simple graphical tool has prompted a fuller discussion in this paper.

The photometric assay. The basic requirement for application of the method is a knowledge of the percentages of the organisms in the culture which grow at certain specified drug concentrations. In principle, only two such points need be provided for a unique "fix" which will permit the prediction of the reaction at all other drug concentrations, although more experimental points may be desirable for higher accuracy.

Some specific applications of the photometric assay in liquid media are discussed in the references cited above. It is assumed that in any new application due attention will be paid to the suitability of the medium, and in particular to its pH. Since the latter affects not only the growth rate in the absence of the antibiotic but, in many instances, has a profound influence on the antibiotic activity, it is important that the medium be sufficiently buffered to minimize variations. Uniform incubation of all tubes in a set is likewise of importance. For the successful application of the graphical method the incubation should be stopped while all tubes are still in the logarithmic growth phase, otherwise a spurious "crowding" of the percentages of

[1] This work was performed under a contract between Yale University and the Office of the Surgeon General, Department of the Army.

[2] Although the word "turbidimetric" has often been applied to such assays, this term is more properly applied to light scattering measurements. The designation "photometric" is preferred here for measurements as ordinarily carried out with an absorption cell in a photoelectric colorimeter even though it is realized that these may involve a scatter component as well.

growth will be obtained, due to a relative slowing of the leading control tube (without antibiotic). An analogous but relatively minor effect occurs even at low densities owing to small pH changes and other variations in the medium, and this prevents direct interpretation of the relative densities as true relative survival percentages. After proper corrections are applied, good agreement with plate counts can, however, be obtained (Muschel and Treffers, 1956*a*, *b*). These considerations should not affect the routine photometric assay of comparative antibiotic activities but must be taken into account in any extended quantitative theory of the process. Only the uncorrected growth percentages will be used here.

Since the graphical plot to be presented is linear only on a logarithmic dosage scale, the amounts of antibiotic or other drug used are most efficiently spread on a logarithmic rather than an arithmetic basis, so that "crowding" of the experimental points may be avoided, over the optimum experimental range (15–85 per cent growth). The closeness of the spacings necessary will vary with the slope of the response curve. In preliminary, "range-finding" experiments a 5-fold or a 10-fold dilution series may be used; even if only one drug concentration results in a readable density, an average slope from previous experiments may be drawn through this point. The resulting curve estimate sometimes suffices for the purpose, or it will permit the setting up of a second, definitive experiment in which the readings for the selected points can be predicted with considerable accuracy.

Graphical expression of photometric assays. Some defects in the usual graphical expressions of assay data are evident from comparative plots. As an illustration, we may consider various representations of a typical titration involving both a sensitive and a moderately drug-resistant organism; separate sets of cultures are incubated for the times indicated, in the presence of appropriate concentrations of the antibiotic. Normally only one incubation time is used for each set, although the time may vary, depending on the growth rate of the culture. The growth levels reached at the end of each period are then measured, the density of the control tube having been checked against a reference growth-time curve to verify that it does not exceed the allowable limit for the logarithmic phase (table 1).

The growth is tabulated in terms of optical density units, obtained on an instrument adjusted to give a density of zero for the uninoculated medium. (If the data are available only in percentages of light transmission they may be converted by the relation: density = 2 − log (per cent transmission.) As is evident, the instrument will be in adjustment to give 100 per cent transmission for the medium blank if the latter units are used). Optical density units are preferable to the percentage transmissions since in the appropriate concentration ranges they are directly proportional to the microbial mass and under standardized conditions, to microbial numbers, whereas as noted, the light transmission involves an additional logarithmic relationship which often obscures regularities apparent when the optical density is used.

As the first method of representation, a part of the data of table 1 may be plotted according to Jackson and Finland (1951), with percentage transmission, and the drug concentrations, as parameters (figure 1). The difference in drug sensitivity of the two strains is well brought out by this means, but the acute curvature is difficult to establish with accuracy, especially for extrapolative predictions, from the few experimental points available here. The various incubation times also result in a family of curves

TABLE 1

Titration against streptomycin (SM) of Escherichia coli and of one of its SM-resistant variants, at various incubation times

SM μg/ml	Parent Culture			SM-resistant culture	
	Incubation time (min)			Incubation time (min)	
	205*	190	231	205*	231
0	0.54	0.31	0.65	0.29	0.34
5	0.52	0.28	0.69	0.29	
10		0.26	0.55		
20			0.47		
40		0.14	0.26		
50	0.17			0.27	
80		0.05	0.11		
100					0.29
160		0.01	0.03		
200	0.02			0.22	0.23
400					0.13
800					0.07

Readings are given in optical density units.
* Preliminary, range-finding experiments.

whose relationships are not immediately evident.

Much of this may be avoided by plotting the data according to the method of Joslyn and Galbraith (1950), in which growth is measured at each level of drug, and the values, in optical density units, are then divided by the optical density of the inoculated control tube which lacked drug. When these ratios are multiplied by 100 there are obtained the (apparent or uncorrected) percentages of growth, which are found to vary with the concentration of drug. If these percentages of growth are plotted against the dilutions of the drug a sigmoidal curve is obtained which is approximately linear over part of the range (figure 2). It is stated by Joslyn and Galbraith that by standardizing the conditions, which include incubation of the titration set until the control tube reaches a predetermined density, the curve can be reproduced on successive days to an average deviation of a ± 5 per cent for the various points. The coincidence here of percentage growths obtained for different incubation times indicates that this restriction is not always necessary.

Valuable as this procedure is, it still suffers from the fact that for our representative data the two lines obtained, while parallel, are not truly linear, the curvature increasing continually away from the 50 per cent point. This latter property hinders the direct analytical comparison of two organisms of different growth rates, or the setting up of a more definitive experiment from preliminary experiments which often include readable data at only one point.

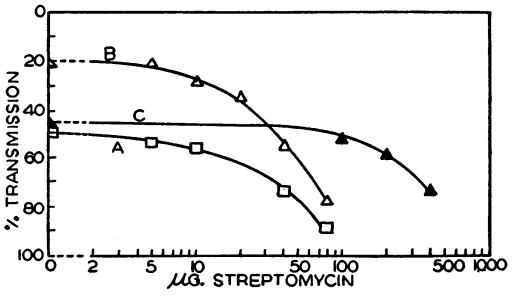

Figure 1. Plot of data for table 1 according to method of Jackson and Finland (1951). Curve *A*, parent culture, incubation time 190 min. Curve *B*, 231 min. Curve *C*, resistant culture, 231 min. Note discontinuity in scale near origin.

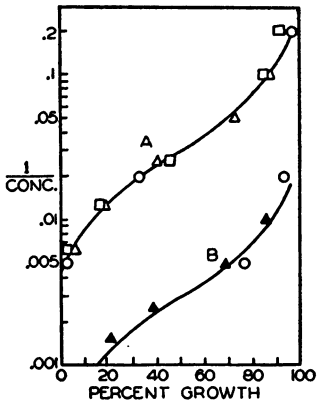

Figure 2. Plot of data for table 1 according to method of Joslyn and Galbraith (1950). Curve *A*, parent culture. Curve *B*, resistant culture. Incubation times: Squares, 190 min; circles, 205 min; triangles, 231 min. Smooth curves drawn independently, via interpolated points (not shown) from figure 3.

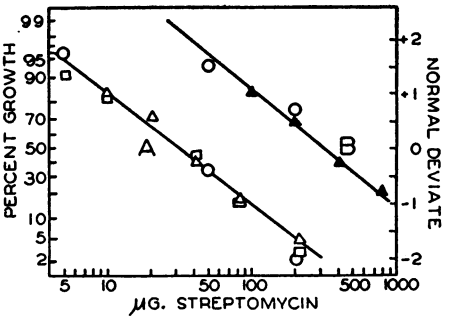

Figure 3. Plot of data for table 1 by normal-curve method proposed in text. Symbols as in figure 2.

Log-normal coordinates. We have found that these difficulties may be eliminated in simple fashion by plotting the same parameters, growth percentages and drug concentrations, not on arithmetic-logarithmic scales but on certain scales involving the special properties of the normal distribution curve (figure 3).

The resulting curves are generally linear over a wide range; for the present data this varies from 3 to 96 per cent growth, with no point deviating from the line by more than 6 per cent and with an average deviation considerably less. The data for the various incubation times, which were sufficiently different to result in final growth densities 100 per cent apart, are also coincident.

Plots of this type are conveniently made on any of several available graph papers.[3] These are ruled with spacings (normal-curve deviate units) such that points corresponding to a normal distribution curve, in its sigmoidal form, will give a linear plot. Conversely, the linearity of the plot on these scales is evidence that the distribution is a normal one. Some papers carry, additionally, the related probit scale.[4] All papers are indexed in percentages, as well, so that the experimental percentages of growth may be plotted directly without further calculation, or reference to tables. The corresponding drug concentrations are inserted on the logarithmically spaced coordinates. Papers are available in two or three log cycles, which suffice for most purposes; if additional dosage cycles are needed any number of sheets may be pasted together.

It will be noted that the percentage scales are symmetrical about the 50 per cent point. Since the spacings increase quite considerably toward the ends, a relatively small and constant experimental error will cause the more extreme points (below 10 or above 90 per cent growth) to deviate disproportionately in actual distance from the true line. This should be taken into account in fitting the line to the experimental points and data in these regions should be given less weight since the experimental error is also likely to be greater for such points. Because of this symmetry about the center, the normal coordinate range cannot be increased as for the logarithmic coordinate by adding additional sheets. The limits usually provided—0.1 to 99.9 per cent—are, however, more than adequate for most purposes, and when measurements of exceptional precision at the extremes become available they can be handled on "extreme value" paper (such as Codex paper no. 3229).

[3] Such as logarithmic normal papers (Codex Book Co., Norwood, Mass., papers 31.376, 32.376, or 3228) or logarithmic probit paper (Keuffel and Esser, N. Y., paper no. 358-22).

[4] The probit is simply the normal deviate unit increased by a value of five, to avoid negative signs in analytical computation. For the present purposes the two may be used interchangeably. The probit is only mentioned here because much of the recent statistical literature is more specifically concerned with it than with the normal deviate unit.

DISCUSSION

The methodology presented is based on nothing more than the observation that the growths in a group of microbial cultures exposed to logarithmically graded increments of drug conform to a certain regularity, which is expressed by the normal distribution curve. By the analytical properties of the latter, simplified here to graphical procedures, empirical applications can readily be made to some practical problems.

Advantages of the log-normal plot. (a) The distribution is generally linear throughout the entire experimental range; in practice, because of experimental difficulties, this is usually 5 to 95 per cent of the control growth. (b) A straight line is, of course, the easiest curve to fit through any set of points; the fitted line represents the best average estimate of the characteristic under investigation; moreover, each point contributes to this average, without wastage of experimental data. (c) Accurate predictions as to the amount of drug necessary to cause, say, 80 per cent inhibition may be made by extrapolation from measurements made outside of this range, for example, in the region 20 to 50 per cent growth. (d) A ready tool is available for the quantitative analysis of combined actions, such as those of two antibiotics, or of an antibiotic and a serum bactericidal system. (e) In addition to the position (ordinate intercept of a 50 per cent endpoint) of the line, this representation offers a new parameter—the slope—without requiring additional experimental data. (f) This design facilitates certain types of statistical analyses, should these be desired.

Endpoint measures. The photometric growth assay is obviously dependent on the average resistance of the culture since the occasional exceptionally resistant organism which may be present will not contribute significantly to the total density in the limited time allowed. This average resistance may be measured by the algebraic equation for the recession line, i.e., its slope and intercept, or more directly, the point of 50 per cent growth may be read off from the scale. The relative potencies of two drugs, or the comparative resistances of two organisms against a single drug, may thus be expressed in terms of the difference between such 50 per cent endpoints, provided only that the slopes be equal in such cases; furthermore, this difference

is often the same whether corrected or uncorrected percentages of growth are used, the correction entering as a constant factor in each, which then cancels (Muschel and Treffers, 1956*a*). Justification for the use of 50 per cent (or other measures within the range 10 to 90 per cent), rather than extreme values which are highly subject to fluctuations, is given in the statistical literature (Finney, 1952).

Significance of slope variations. The slope of the response line is inversely proportional to the standard deviation, i.e., the spread of the corresponding normal distribution curve. Its significance may be better visualized by a specific case. The slope of each of the lines of figure 3 is 2.10. It is derived by dividing the change in normal deviate units (as read on the scale opposite to the growth percentages plotted) for a given increase in the dosage measured in log units, or, more simply, by measuring the change in normal deviate units (here, 2.10) for any 10-fold increase in dosage (i.e., one log unit). By inspection of the curve it may be seen that with this slope the antibiotic concentrations which result in growths of, say, 80 and 20 per cent, are 11 and 80 μg, respectively, a concentration ratio of 7.3. For any parallel line (increased or decreased susceptibility) there will, of course, be a change in intercept (or 50 per cent endpoint) although, by definition, not of slope. The absolute concentrations for 80 and 20 per cent growths will therefore change but, significantly, the concentration ratio will not. This case is frequently noted for pairs of susceptible organisms and the drug-resistant variants derived from them (Treffers *et al.*, 1953–54). On the other hand, for a line of the same 50 per cent endpoint, 31 μg, but with a slope of 3.0, the concentrations for 80 and 20 per cent growth would be 16.5 and 59 μg, a concentration ratio of 3.6. A difference in slope thus requires that the concentration of one drug will have to be increased, disproportionately, over the other to effect equal further increases in inhibition. If the slope is relatively low it may be possible to effect some inhibition of the organisms only at low drug concentrations; further inhibition requires impossibly high drug concentrations. Similar considerations apply to changes of slope with varying specificities of antibodies (Muschel and Treffers, 1956*c*).

Slope variations have several other implications. (1) It is evident from the above that unless the response curves for two drugs have the same slope, the potency difference between them cannot be expressed in general by any single numerical value, but requires a qualification of the degree of inhibition at which the comparisons are made. With certain combinations of slopes and intercepts (as above) a complete reversal in potency with concentration may occur so that the drug which was the more effective at low degrees of inhibition becomes the weaker member for higher degrees of inhibition. Thus, equality of slope is assumed in the usual measurement of antibiotic potency differences, whether warranted or not by experimental fact.

(2) Two drugs acting on the same species of organism, or one drug acting on two species, may, by coincidence, exhibit response curves of identical slope without warranting the conclusion that the mechanisms of action are identical, or even related in any way. A change in slope, with change in organism or drug, does, however, denote a change in the distributions of susceptibilities and is at least highly suggestive that some alteration in the mechanism of action has occurred.

Slope measurements therefore represent real gains in information, without requiring additional experimental data.

Statistical procedure. Except for special purposes, little or no additional statistical control is needed. The adherence of most experimental points to a linear relationship is sufficiently good so that a line fitted by an "eye-estimate" will serve; if desired, however, an analytical fit may be made with weighted probits, and the method of least square (Finney, 1952). As has been noted above, the standard deviation of the distribution of logarithmic tolerances is given directly by the reciprocal of the slope of the line. If further desired, the standard error of the 50 per cent endpoint may be calculated through the general formula of the standard error of a ratio (Quenouille, 1952). In instances where two regression lines do not appear to be parallel, an objective analysis for significance of the difference may be made by calculating the joint or combined regression, followed by an analysis of variance (Quenouille, 1950). Further information on these problems is given by Finney (1952); an application to a photometric bactericidal

assay is given by Muschel and Treffers, 1956*a*.

Microbiological implications of the linear relationship. At least three hypotheses may be offered to account for the observed normal distribution of growths, on the assumption of a large number of organisms which may differ from each other in the lag time before multiplication ensues, or the growth rate once multiplication has begun, or both.

(1) In the presence of increasing amounts of drug only a diminishing percentage of the organisms inoculated can grow at all, the number being governed by the normal distribution curve. Some basis for this has been provided in the plating experiments reported by Demerec (1948), and later workers, if it is further assumed that all of the organisms capable of growth at a given drug concentration do so with uniform lag and growth rates.

(2) All of the organisms grow, with constant growth rates, but with a distribution of lag times governed by the normal curve.

(3) All of the organisms grow, with uniform lags, but with growth rates governed by the normal distribution curve.

Experiments designed to decide among these hypotheses are now under way, and will be reported later. From the preliminary results it would appear that no one of these possibilities operates uniquely or even uniformly in all cases, and that many instances may involve complex alterations in both the lag and the growth rate which are qualitative as well as quantitative functions of the drug and of its concentration. In at least some instances, significant reductions in growth in the presence of the antibiotic do not involve any inheritable increase in resistance for the majority of the organisms. This can be demonstrated by the retitration of a culture selected from the 10 per cent growth tube of an experiment such as that of figure 3; curves identical with that of the original culture have been obtained. Finally, the coincidence of points obtained at different incubation times (figure 3) would argue against hypothesis (3) above, since the latter should result in lines which increase in slope with increasing incubation time.

SUMMARY

A simple graphical procedure is described whereby the results of photometric growth assays of antibiotic activity may be conveniently represented by plotting the percentages of growth as ordinate on a normal curve-deviate scale, against the logarithms of the antibiotic concentrations. In many instances this representation will be linear from 2 to 98 per cent of growth (98 to 2 per cent inhibition). This linearity permits ready interpolation or extrapolation of data, or the assessment of a 50 per cent endpoint. In favorable instances the entire dosage-response curve can be predicted from only two experimental measurements, or a single measurement and knowledge of the average slope for such systems. The slope of the line also affords a useful parameter which enters into the assessment of potency differences or serves as an indication of possible changes in mechanisms of action. Utilization of this plot also facilitates certain statistical examinations of the data.

REFERENCES

DEMEREC, M. 1948 Origin of bacterial resistance to antibiotics. J. Bacteriol. **56**, 63–74.

FINNEY, D. J. 1952 *Probit analysis.* ed. 2, University Press, Cambridge.

HEATLEY, N. G. 1949 The assay of antibiotics. Ch. 3 in *Antibiotics*, ed. Florey and others. Oxford University Press, New York and London.

JACKSON, G. G. AND FINLAND, M. 1951 Turbidimetric method for determining the sensitivity of bacteria to antibiotics. J. Lab. Clin. Med., **38**, 289–307.

JOSLYN, D. A. AND GALBRAITH, M. 1950 A turbidimetric method for the assay of antibiotics. J. Bacteriol., **59**, 711–716.

MAALØE, O. 1946 *On the relation between alexin and complement.* Einar Munksgaard, Copenhagen.

MUSCHEL, L. H. AND TREFFERS, H. P. 1956*a* Quantitative studies on the bactericidal actions of serum and complement. I. A rapid photometric growth assay for bactericidal activity. J. Immunol. **76**, 1–10.

MUSCHEL, L. H. AND TREFFERS, H. P. 1956*b*. Quantitative studies on the bactericidal actions of serum and complement. II. Some implications for the mechanism of the bactericidal reaction. J. Immunol. **76**, 11–19.

MUSCHEL, L. H. AND TREFFERS, H. P. 1956*c* Quantitative studies on the bactericidal actions of serum and complement. III. Observations on sera obtained after T.A.B. vaccination or during typhoid fever. J. Immunol. **76**, 20–27.

QUENOUILLE, M. H. 1950 *Introductory statistics.* Butterworth-Springer, London.

QUENOUILLE, M. H. 1952 *Associated measurements*. Academic Press, New York.

TREFFERS, H. P., BELSER, N. O., AND ALEXANDER, D. C. 1953–54 Genetic studies on resistance to streptomycin and to a substituted streptomycin (N′-γ-hydroxypropylstreptomycylamine). Antibiotics Annual, 595–603 Medical Encyclopedia, N. Y.

TREFFERS, H. P. AND MUSCHEL, L. H. 1954 The combined actions of chloramphenicol and of bactericidal antibody plus complement on *Salmonella typhosa*. J. Exptl. Med. **99**, 155–165.

WAKSMAN, B. H. 1949 A comparison of the von Krogh formula (logistic function) and the method of probits as applied to hemolyses by complement. J. Immunol., **63**, 409–414.